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in Plant Systems and Initial Assessment....

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EVALUATION OF THE METABOLIC FATE OF MUNITIONS
MATERIAL (TNT & RDX) IN PLANT SYSTEMS AND INITIAL
ASSESSMENT OF MATERIAL INTERACTION WITH PLANT GENETIC
MATERIAL

VALIDATION OF THE METABOLIC FATE OF MUNITIONS MATERIALS
(TNT, RDX) IN MATURE CROPS

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FOREWORD

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EXECUTIVE SUMMARY

The goals of this effort were to confirm and expand data related to the behavior and impacts of munitions residues upon human food chain components. Its objective was to assess relative accumulation/partitioning, and chemical form(s) of munitions material within key crop plants so that prior U.S. Army Biomedical Research and Development Laboratory (USABRDL) sponsored research with trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) might be enhanced and further validated. Plant species employed included corn (*Zea mays*), alfalfa (*Medicago sativa*), spinach (*Spinacea oleraceae*), and carrot (*Daucus carota*). Plants were grown from seed to maturity (70 to 120 days) in a low-fertility soil (Burbank) amended with either ^{14}C -TNT or ^{14}C -RDX at which time they were harvested and analyzed for munitions uptake, partitioning, and chemical form of the munition or munition-metabolite. A second study was also performed to assess the impact of differing nitrogen (N) nutrition regimes on the uptake and fate of soil-amended TNT in alfalfa.

All four of the plant species used in this study accumulated the ^{14}C -TNT-derived label and distributed portions of it throughout the entire plant when grown from seed. The study employed a soil TNT concentration of three times that used in the previous work to optimize impact identification. Correspondingly, the specific activities (μg TNT-equivalents/g dry wt.) and total uptake (μg TNT-equivalents) observed in the current study were approximately three times greater.

The carrot, alfalfa, and corn demonstrated a higher percentage of label retained in the roots (62, 73, and 83%, respectively). The spinach contained less activity in its root (36%) but also contained the highest TNT specific activity observed ($>4600 \mu\text{g}$ TNT-equivalents/g dry wt.). The spinach root mass was significantly smaller than the other species tested, and the specific activity values may have been affected by this. However, the ratio of the total label found within the plants/plant total dry wt. for the spinach was twice that of the carrot and three times that of the alfalfa indicating that the spinach may have a higher rate of contaminant accumulation than the other two. Analysis of carrot root cross-sections revealed an even distribution of the TNT-derived label across the root. In the corn plants, the roots contained the highest specific activity, actual total TNT-equivalents, and highest percent of label distribution. The corn leaves were second which may be attributable to the large leaf surface area of corn plants and high rates of xylem transport to these tissues. The low allocation of the label (and hence TNT and TNT-metabolites) to the reproductive parts of the plant (tassels, ear) suggests that these organs would not be a source of contamination in an environmental food chain.

The specific uptake values of RDX for the spinach and alfalfa were comparable to those previously reported for wheat and bean (314 to 590 μg RDX-equivalents/g dry wt., respectively). An exception to this may be the carrot where the specific activity was found to exceed 4200 μg RDX-equivalents/g dry wt. in the shoot with an accompanying tissue concentration of over 4000 μg . In the root, the RDX-derived label was distributed evenly throughout the tissue. Consistent with the other plant species tested, the roots of corn contained only a small part (<6%) of the total label (RDX-equivalents). The leaves were the tissue with the highest specific activity and total amount of RDX-derived label (>75%). This is, again, not surprising considering the apparent mobility of RDX around the plant, the large surface area of the leaves with the accompanying high phloem transport activity and transpiration rate .

Contrary to the TNT plants and similar to the previous species grown in RDX contaminated soil such as bean and grass, reproductive parts of the corn plant (tassels, ear) contained a significant allocation of the label. Given the report by Harvey et al. (1991) and Cataldo et al. (1993), much of the soil-accumulated RDX remains as the parent compound in plant tissues; these dispersible plant parts may become a secondary source of contamination to the environment. This would be particularly true for the pollen contained in the tassels.

The total accumulation of TNT by the plants was only a small portion of the actual labeled compound originally amended to each pot of soil. These total accumulation values ranged from 1.24% for the spinach to 2.34% for the carrot. This was much higher for the RDX plant ranging from 15% for the spinach to 37% for the carrot. This supports the previous observation that RDX is readily bioavailable to plants.

Both TNT and RDX assimilated by higher plants growing in contaminated soil may have differing metabolic fates. The leaves of corn and the shoot of alfalfa were selected for analysis based both on their radiolabel contents and their increased potential as starting points of food chain interactions (silage and hay). Only 2 to 6% of the total label was found in the F2 (nonpolar) fractions of the TNT grown plants with the remainder found in the soluble polar fractions (24 to 52%) or the insoluble pellet (46 to 70%). These results are slightly lower than those seen before but are generally similar in the overall pattern. High Pressure Liquid Chromatography (HPLC) analysis of the F2 fraction, however, indicated no identifiable TNT or aminodinitrotoluene (ADNT) isomers present. This may be attributable to the longer growth period the plants went through in this study (110 to 120 days) compared to the earlier work (60 days). The patterns for the RDX plants were also similar to those observed previously in bean, wheat, and grass. Higher activity existed in the F2 fraction of spinach carrot and alfalfa (up to half of the soluble fraction in the alfalfa stem) and the HPLC analysis identified the parent RDX compound at significant levels in the shoot of alfalfa (>180 $\mu\text{g/g}$) and corn (>18 $\mu\text{g/g}$).

Plants grown in the Burbank soil consistently exhibited higher rates of uptake from the soil of amended munitions material. This was correlated with the soil's relatively low nutritional status. The principal plant nutrient missing from this soil type when compared to the others tested previously was nitrogen (N).

Alfalfa plants were grown in ^{14}C -TNT-amended Burbank soil and control nonamended Burbank in the same growth chambers. Supplemental nitrogen was withheld (plain soil), given as fertilizer, or given as a *Rhizobia* inoculum. Plants growing in the plain soil were very small in stature after 45 days when compared to the supplemental nitrogen treatments. The plants growing in plain soil also exhibited high tissue specific activities in their roots in excess of 14,000 TNT-equivalents/g dry wt. perhaps suggesting a nutrient stress-driven tendency to acquire alternate nitrogen sources from the soil. This data must be taken with caution, however, because the average root dry weight for these plants was 0.02 g and the total activity found in these organs averaged 241 μg ^{14}C -TNT-equivalents compared to the 877 and 927 μg ^{14}C -TNT-equivalents for the *Rhizobia* and fertilizer plants, respectively. No TNT toxicity was apparent in the larger plants provided with a supplemental nitrogen source; further, no significant differences were evident between the supplemental nitrogen treatments in either dry weight, specific activity, tissue contents, or distribution patterns. Therefore, while some circumstantial evidence exists for a TNT stimulation of growth where the nitrogen content of the soil may be limited, definitive proof was not obtained.

The results of this study confirm those performed previously with wheat, grass, and bean. Again, the observations demonstrate that crop plants are capable of accumulating the munitions material and partitioning the material itself or its metabolites within tissues directly consumed by herbivores, or by man himself. The information presented here suggests that the amounts of these parent compounds or their metabolites may reach significantly high levels (e.g., $>180 \mu\text{g/g}$ RDX in alfalfa shoots) even after the plant has been exposed to the material for over 120 days. No information exists as to the toxicity of these potentially bioavailable forms to the primary and secondary consumers. This type of information would be important for the accurate performance of any risk analysis required by federal regulations. The information currently available may force such an evaluation to be excessively conservative and therefore severely restrict remediation options on the part of the U.S. Department of Defense with a concomitant increase in the final costs.

CONTENTS

FOREWORD	vii
EXECUTIVE SUMMARY	ix
1.0 INTRODUCTION	1
1.1 REVIEW OF RELATED LITERATURE	1
1.1.1 Chemistry and Analytic Methods	2
1.1.2 Plant Uptake and Metabolism	2
1.2 TECHNICAL APPROACH.....	5
2.0 MATERIALS AND METHODS	6
2.1 CHEMICALS AND STANDARDS	6
2.2 SOIL CHARACTERIZATION AND SAMPLING	6
2.3 PLANT CULTIVATION	7
2.4 CHEMICAL AND ANALYTICAL PROCEDURES.....	8
2.4.1 Radioanalyses.....	8
2.4.2 Tissue Extraction and Fractionation	8
3.0 RESULTS AND DISCUSSION	11
3.1 DISTRIBUTION OF MUNITIONS RESIDUES IN PLANT TISSUES AT MATURITY	11
3.1.1 TNT Uptake and Distribution	12
3.1.2 RDX Uptake and Distribution.....	18
3.2 CHEMICAL ANALYSIS OF INDIVIDUAL TISSUES FROM SOIL- GROWN PLANTS	23
3.3 DETERMINATION OF ALTERATIONS IN PLANT PARTITIONING WITH VARIATIONS IN PLANT NUTRITIONAL STATUS	26
4.0 SUMMARY AND CONCLUSIONS	29
4.1 DISTRIBUTION OF MUNITIONS RESIDUES IN PLANT TISSUES AT MATURITY	29
4.1.1 TNT Uptake and Distribution	29
4.1.2 RDX Uptake and Distribution.....	30
4.2 CHEMICAL ANALYSIS OF INDIVIDUAL TISSUES FROM SOIL- GROWN PLANTS	31
4.3 DETERMINATION OF ALTERATIONS IN PLANT PARTITIONING WITH VARIATIONS IN PLANT NUTRITIONAL STATUS	31
4.4 RESEARCH NEEDS	32
5.0 LITERATURE CITED.....	34

FIGURES

2.1	Flow Chart for the Analysis of TNT in Plant Tissues.....	9
2.2	Flow Chart for the Analysis of RDX in Plant Tissues.....	10

TABLES

2.1	Selected Properties of Burbank Test Soil	7
3.1	Specific Uptake ($\mu\text{g } ^{14}\text{C-TNT}$ Equivalents/g dry wt.) in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 30 $\mu\text{g/g } ^{14}\text{C-TNT}$	13
3.2	Dry Weights (g) of Alfalfa, Spinach, and Carrot Plants Grown for 70 to 90 Days on Burbank Soil Alone (Control) or Amended with 30 $\mu\text{g/g } ^{14}\text{C-TNT}$ (Amended)	14
3.3	Whole Tissue Concentrations of $^{14}\text{C-TNT}$ -Equivalents in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 30 $\mu\text{g/g } ^{14}\text{C-TNT}$	15
3.4	Percent Distribution of Radiolabel in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 30 $\mu\text{g/g } ^{14}\text{C-TNT}$	16
3.5	Specific Uptake ($\mu\text{g } ^{14}\text{C-TNT}$ -Equivalents/g dry wt.), Total Tissue Content ($\mu\text{g } ^{14}\text{C-TNT}$ -Equivalents), and Percent Distribution of Radiolabel in Corn Plants Grown for 100 to 120 Days on Burbank Soil Amended with 30 $\mu\text{g/g TNT}$	18
3.6	Specific Uptake ($\mu\text{g } ^{14}\text{C-RDX}$ -Equivalents/g dry wt.) in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 15 $\mu\text{g/g RDX}$	20
3.7	Dry Weights (g) of Alfalfa, Spinach, and Carrot Plants Grown for 70 to 90 Days on Burbank Soil Alone (Control) or Amended with 15 $\mu\text{g/g } ^{14}\text{C-RDX}$ (Amended)	20
3.8	Whole Tissue Concentrations of $^{14}\text{C-RDX}$ -Equivalents of Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 15 $\mu\text{g/g } ^{14}\text{C-RDX}$	21
3.9	Percent Distribution of Radiolabel in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 15 $\mu\text{g/g } ^{14}\text{C-RDX}$	22
3.10	Specific Uptake ($\mu\text{g } ^{14}\text{C-RDX}$ -Equivalents/g dry wt.), Total Tissue Content ($^{14}\text{C-RDX}$ -Equivalents), and Percent Distribution of Label in Corn Plants Grown for 100 to 120 Days on Burbank Soil Amended with 15 $\mu\text{g/g RDX}$	23

3.11	Percentage of Total Radioactivity in the Various Chemical Fractions of Alfalfa Stems from Plants Grown to Maturity (90 to 120 days) in ^{14}C -TNT (30 $\mu\text{g/g}$ dry wt soil) or ^{14}C -RDX (15 $\mu\text{g/g}$ dry wt. soil) Amended Burbank Soil.....	24
3.12	Percentage of Total Radioactivity in the Various Chemical Fractions of Corn Leaves from Plants Grown to Maturity (90 to 120 days) in ^{14}C -TNT (30 $\mu\text{g/g}$ dry wt. soil) or ^{14}C -RDX (15 $\mu\text{g/g}$ dry wt. soil) Amended Burbank Soil.....	25
3.13	Specific Activity ($\mu\text{g } ^{14}\text{C}$ -TNT equivalents/g dry wt. tissue), Total Tissue Contents ($\mu\text{g } ^{14}\text{C}$ -TNT Equivalents), and Percent Label Distribution in Alfalfa Grown under Differing Nitrogen Treatments	27
3.14	Dry Weight (g) of Alfalfa Plants Grown for 45 days in Burbank Soil Amended With 15 $\mu\text{g/g } ^{14}\text{C}$ -TNT and Provided Different Nitrogen Treatments	28

1.0 INTRODUCTION

Munitions material such as trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and their combustion and decomposition products can enter the environment as a result of production and manufacturing activities as well as field usage and disposal (Small and Rosenblatt 1974; Spanggord et al. 1983; Ryon et al. 1984). Once released, these contaminants will undergo chemical partitioning that is dependent both on sorptive processes in the soil and water solubility (Bell 1992) in the soil pore water and ultimately the groundwater. Therefore in assessing the potential environmental risk of these residues, the mere presence of specific munitions-related products in the environment cannot be indicative of the presence or severity of an impact. Both the relative accessibility to subsequent dependent components in the food chain (e.g., partitioning to commonly edible tissues) and characteristic deleterious effects (i.e., chronic damage patterns) of these products within the biotic components of terrestrial and aquatic environs must be considered. For terrestrial systems, a critical link is accumulation by green plants, the fundamental component of the terrestrial food chain.

The goals of the effort described in this report were to confirm and expand data related to the behavior and impacts of munitions residues released to the environment via human food chain components. This study represented a follow-on inquiry based on the results of recently completed activities (TNT, RDX) sponsored by U.S. Army Biomedical Research and Development Laboratory (USABRDL). The objective of this study was to validate prior USABRDL-sponsored research with TNT and RDX using mature field crops. Activities assessed the relative accumulation, partitioning, and chemical form(s) of munitions material within key crop plants expected to be vital food chain components associated with operating or released production facilities.

1.1 REVIEW OF RELATED LITERATURE

While several studies have been conducted on the development of analytical methods and subsequent characterization of parent compounds and decomposition products associated with waste streams, impoundments, and/or releases from production sites, few have dealt with the environmental persistence, bioavailability, and metabolic detoxification of these materials. Even fewer have addressed chemical fate and behavior at environmental concentrations within the solubility constraints of the munitions components.

1.1.1 Chemistry and Analytic Methods

Effluent waste streams associated with TNT production processes have been carefully characterized. The streams have been shown to contain over 30 isomeric nitroaromatics and associated byproducts (Spanggord et al. 1982) that ranged in concentration from 0.001 to 48 mg/L of ether-extracted effluent. Substantially less work has been performed with respect to RDX decomposition products. Spanggord et al. (1983) have determined that RDX is subject to limited photochemical and soil microbial attack with photochemical effects including cleavage of the nitro groups and the subsequent formation of hydrazine. Chemical hydrolysis resulted in the reduction of nitro groups. No evidence of significant ring opening is available for TNT or the cyclonites.

Established methodologies are required to ascertain both chemical and biologically based transformations. A number of gas chromatograph (GC) and gas chromatograph/mass spectrometer (GC/MS) methods are available to permit identification and quantification of parent compounds, by-products and decomposition products (Spanggord et al. 1982, 1983; Belkin et al. 1985). Previous applications which involved high pressure liquid chromatography (HPLC), were suitable only for resolution and quantification of major constituents (Jenkins et al. 1986; Bauer et al. 1986) in soils (Jenkins and Grant 1987) and plant tissues (Palazzo and Leggett 1986) extraction methods. More recently, work at the Pacific Northwest Laboratory¹ (Cataldo et al. 1989, 1990; Harvey et al. 1990, 1991, 1992; and Fellows et al. 1993a, 1993b) has permitted development of new extraction methodologies for soil and plant tissues exposed to TNT, RDX, and tetryl, with the subsequent analysis and identification of metabolites through HPLC and GC/MS.

1.1.2 Plant Uptake and Metabolism

Until recently, the literature contained very little data related to soil and plant fate and bioavailability of TNT, RDX, and tetryl. In the 1970s, a substantial amount of research was performed on aquatic organisms including algae and water plants. TNT has been found to be toxic to duckweed at levels in excess of 1 mg/L (Schott and Worthley 1974), to inhibit freshwater algae growth at 2 to 15 mg/L (Smock et al., 1976), and to inhibit the growth and metabolism of microorganisms (Klausmeier et al. 1973; Nay et al. 1974). With the exception of Smock et al. (1976) and Schott and Worthley (1974), no chemistry was performed on either culture solutions or materials accumulated. The latter authors, however, did note a conversion of TNT to 2,4-

¹ Pacific Northwest Laboratory is operated for the U.S. Department of Energy by Battelle Memorial Institute under contract DE-AC06-76RLO1830.

dinitrotoluene (2,4-DNT) and 4-amino-2-Nitrotoluene (4-amino-2-NT) in their culture medium.

One higher plant study was performed using hydroponically grown yellow nutsedge to access the uptake, toxicity, and metabolic transformations of TNT (Palazzo and Leggett 1986). This study showed shoot and particularly root growth to be inhibited at 5 mg/L. Although not noted by the authors, these toxicity symptoms are characteristic of dinitroaniline herbicide damage. The latter would be expected based of the chemical structure of TNT and particularly the aminodinitrotoluenes. Chemical analysis showed >90% of all extractable tissue material to be present as 2- and 4-aminodinitrotoluene (2- and 4-ADNT), with only a small amount of TNT being recovered. Because these species were not observed in the nutrient solutions, they are assumed to be metabolic detoxification products. No data were found for RDX or tetryl.

Detailed plant fate studies related to TNT and RDX were conducted (Cataldo et al. 1989, 1990; Harvey et al., 1990, 1991). Plants were found to have TNT-derived ^{14}C localized primarily in the roots, with a small amount of radiolabel being transported to the shoot and leaves. Studies examining the xylem exudate of bush beans grown in TNT-containing hydroponic solutions allowed for identification of the primary transport forms of TNT. Acid hydrolyzed xylem exudate was found to contain 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene, as well as an unidentified TNT metabolite. TNT is transported as these polar conjugates to the aerial portions of the plant where it undergoes further metabolic alteration. Studies indicated that the polar TNT metabolites are sequestered within the plant potentially accumulating at sites where they could be consumed and are not transpired as volatile organics or $^{14}\text{CO}_2$. Chemical fractionation of plant tissues grown in TNT-containing hydroponic solution indicated that less than 12% of the incorporated radiolabel was contained in TNT, 2-amino-4,6-dinitrotoluene, or 4-amino-2,6-dinitrotoluene. The majority of the radiolabel was found in either a polar ether-extractable fraction, or in a more polar nonether extractable fraction. The preponderance of these previously unknown, highly polar TNT transformation products, was a major finding of an earlier study (Fellows et al. 1993b).

The explosive RDX, on the other hand, was found to be bioaccumulated in the aerial tissues of the plants studied (Cataldo et al. 1990; Harvey et al. 1991; Banwart et al. 1991). Studies with plants grown to maturity on RDX-amended soils show RDX-derived metabolites to accumulate in all tissues (Cataldo et al. 1990). The relative order of tissue concentration is seed > leaves \geq stem > root > pod. The tissue concentrations in these plants for RDX-derived residues at maturity were as high as 200 and 600 $\mu\text{g/g}$ fr. wt. for leaves and seed, respectively. Roots

contained less than 75 µg/g fr. wt. In wheat and blando brome, leaf concentrations of RDX were as high as 550 µg/g fr. wt., while roots contained less than 45 µg/g fr. wt. Concentrations of RDX-derived residues in the grass seed were as high as for leaves. Calculation of the percentage removal of RDX from the pots by single plants based on biomass production and tissue concentrations indicate that 11 to 55% of the soil RDX was removed by bush beans, and 10 to 40% was removed in a single harvest of wheat and blando brome. Tissue partitioning and chemical analyses indicate that RDX is the only nonpolar metabolite isolated from plants grown to maturity. RDX accounts for 6 to 53% of the accumulated residue activity in wheat and blando brome, 6 to 12% in bush bean leaves, and 2 to 10% in bean pods. Most of the remaining activity found within the plants was in unidentified polar metabolites. This indicates that the metabolism of the RDX is much slower than that of TNT and that this may vary between species.

Results for plants grown in 25 mg/L ¹⁴C-tetryl amended soil (Fellows et al. 1993a) indicated significant ($P < .01$) differences in the total relative uptake of tetryl by three separate plant species based on soil type. Bush bean plants grown in the Burbank soil accumulated twice as much tetryl/g fr. wt. as those grown in the Palouse soil (74 vs. 147 µg/g fr. wt.) and four to five times that of the plants grown in the Cinebar soil (32 vs. 147 µg/g fr. wt.) (Fellows et al. 1993a). Similar uptake differences were evident in the blando brome (48 vs. 115 µg/g fr. wt. for Burbank versus Palouse), but this trend was not as pronounced in wheat (58 vs. 89 µg/g fr. wt., respectively). These observations are similar to those reported for TNT and RDX (Cataldo et al. 1989, 1990) where such differences were attributed to the soil organic matter content and cation exchange capacity (CEC).

Analysis of the tissue distribution patterns for tetryl indicated that tissue partitioning for mature plants grown on soil differed from that observed in short-term hydroponic studies. While the root remains the primary repository of accumulated tetryl residues, the shoots accumulated substantially more of the residues. For bush beans, the roots contained ~80% of the label, the leaves ~10%, the stems ~7%, and the seed and pod ~3%. In wheat and blando brome, 16 to 20% of the accumulated label was found in the shoot tissues. This would indicate that significant transport of tetryl residues to plant shoots can occur over their life cycle. This pattern is very similar to that of TNT (a structurally similar compound) where 65 to 75% was retained within the roots and different from the RDX where the label was distributed evenly throughout the plant.

The chemical analytical results of these soil and, particularly, plant studies have demonstrated that compounds such as TNT and RDX show high bioavailability, but more

importantly, chemical transformation and sequestration. Thus, the results of small-scale laboratory studies must be validated for representative field crops, and that the fate of sequestered metabolites be investigated.

1.2 TECHNICAL APPROACH

The following report will address uptake and partitioning of munitions material (TNT and RDX) in four species of crop plants which are known components of the human food chain potentially found near contaminated sites. Plant species employed include corn (*Zea mays*) a grain plant; alfalfa (*Medicago sativa*), a forage legume capable of atmospheric nitrogen fixation; spinach (*Spinacea oleraceae*), a vegetable crop harvested for the foliar portion of the plant; and carrot (*Daucus carota*), a vegetable harvested for its below-ground portion or storage root. These provide representative monocotyledon and dicot seed sources, a typical browse species for evaluation of direct and indirect food chain pathways to humans, and vegetable crops important for either their above- or below-ground portions. The soil employed is Burbank silt-sand. Because of the soil's lower CEC and organic matter content, plants grown in the soil have consistently exhibited the highest accumulation rate of the munition's material for those soil types tested (Cataldo et al. 1989, 1990; Fellows et al. 1993a).

2.0 MATERIALS AND METHODS

Much of the materials and methods employed in this work have been previously described in Cataldo et al. (1989, 1990), Harvey et al. (1990, 1991), and Fellows et al. (1993a).

2.1 CHEMICALS AND STANDARDS

A series of standards including 2,4,6-trinitrotoluene, 2-amino-4,6-dinitrotoluene, and 4-hydroxylamine-2,6-dinitrotoluene were obtained from Chem Service (West Chester, PA), U.S. Army THAMA SARM Repository, and Oak Ridge National Laboratory, respectively. Purity was >98% for each compound. Uniformly ring-labeled ^{14}C -TNT (specific activity of 5.3 mCi/mM) was obtained from E.I. du Pont de Nemours & Co. (Boston, MA). Radiopurity, based on HPLC radiochromatography, was 99.86%

Acetonitrile (ACN) solutions of RDX were obtained from Battelle Columbus Division (West Jefferson, Ohio), and the RDX recrystallized from the acetone solution. The purity of the recrystallized product was >99.8%. Uniformly ring-labeled ^{14}C -RDX (specific activity of 25.3 mCi/mM) was obtained from Sigma Chemical Company (St. Louis, MO). The radiolabeled RDX was examined, and its purity determined by radiochromatography. A subsequent purity of 90.5 % was calculated which was not sufficient for metabolic studies. Following further purification as described in Cataldo et al. (1990), the final purity was determined as 99.93 %. This purity was judged appropriate for metabolic studies.

2.2 SOIL CHARACTERIZATION AND SAMPLING

Previous studies have indicated that there is an inverse relationship between the amount of munitions material taken up by the plant and the CEC/organic matter content of the soil (Cataldo et al. 1989, 1990; Fellows et al. 1993a). Therefore, Burbank, a soil type previously demonstrated to give the highest plant uptake rate (Fellows et al. 1993a) was used.

Burbank is a sandy loam (sandy, skeletal, mixed xeric Torriorthent) and is representative of the desert areas of Washington, Oregon, and Idaho. The soil was collected on the Hanford Reservation, WA, and consisted of the Ap horizon. This soil is 51.4% silt, and 4.0% clay, 45.1% sand, contains 0.5 % organic matter. Also the soil has a CEC of 5.5 meq/100 g and a pH of 7.4. The chemical and physical characteristics of this soil are presented in Table 2.1.

TABLE 2.1. Selected Properties of Burbank Test Soil.

Soil Property	Burbank Sandy Loam
Sand (%)	45.1
Silt (%)	51.4
Clay (%)	4.0
Ash (%)	98.0
pH (100% field capacity)	7.4
Organic carbon (%)	0.5
Sulfur (%)	0.0
Nitrogen (%)	0.061
Total P (µg/g)	2400.0
Phosphate-P (µg/g)	4.8
Carbonate/Bicarbonate (%)	<0.1
Ammonium-N (µg/g)	6.1
CEC (meq/100g)	5.5

For soil experiments, a solution containing appropriate proportions of labeled and/or unlabeled TNT or RDX was prepared in 2.0 mL of methanol (TNT) or acetone (RDX) and amended to pre-weighed quantities of air-dried soil to give final concentrations described in Sections 3.1 and 3.3. Control soils were amended with the solvent carrier alone. Soils were brought to 66% of field capacity with water for 24 h before amendment and then maintained at this moisture level throughout the experiment.

2.3 PLANT CULTIVATION

Plant species employed included corn (*Zea mays*), alfalfa (*Medicago sativa*), spinach (*Spinacea oleraceae*), and carrots (*Daucus carota*). All plants were grown from seed. Spinach, alfalfa, and carrots were grown in plastic-lined pots containing 800 g of soil (dry wt.) while the corn was grown in plastic-lined pots containing 4000 g of soil (dry wt.). Seed were planted into amended and control pots approximately 72 h following amendment to permit evaporation of carrier solvent before planting.

All plants were maintained in controlled-environment chambers with a 12/12-h light/dark cycle ($500 \mu\text{E m}^{-2} \text{sec}^{-1}$, Photosynthetically Active Radiation [PAR], at leaf surface), at a day/night temperatures of 26/22°C, and 50% relative humidity. With the exception of the fertility study described in Section 3.3, all plants were watered three times weekly with distilled water. In addition, the spinach, alfalfa, and carrots were watered twice monthly with 50 mL per pot of a fertilizer solution (0.005% w/v of Peters Professional Water Soluble Fertilizer, 20-20-20; [W.R. Grace and Co., Fogelsville, PA]). The corn was watered monthly with the fertilizer solution at a rate of 500 mL per pot.

2.4 CHEMICAL AND ANALYTICAL PROCEDURES

2.4.1 Radioanalyses

Upon reaching maturity, the plants were harvested by depotting the plants, gently removing the loose soil from the root masses, and then washing the excess soil from the roots in successive rinses of distilled water, 80% (v/v) methanol, and 0.1M CaCl_2 . The roots were then blotted dry, the plant was divided into segments (described in the text), and a fresh weight was taken. The tissue was then frozen in powdered dry ice and freeze-dried. Tissues were subsequently weighed, ground in a Wiley Mill (VWR Scientific, Seattle, WA) (20 mesh screen) and aliquotes oxidized by total combustion in a Packard Model 307 oxidizer (Packard Instrument Co., Meriden, CT) to determine the amount of radiocarbon associated with each sample. Combusted samples and liquid samples were counted using a Beckman 9800 Liquid Scintillation Spectrometer (Beckman Instruments, Downers Grove, IL) with appropriate quench correction.

2.4.2 Tissue Extraction and Fractionation

Randomly selected plants in each experiment were harvested, frozen in dry ice and then maintained at -80°C for analysis of TNT and RDX distribution and metabolism. Extraction schemes previously developed for TNT (Cataldo et al. 1989; Harvey et al. 1990) and RDX (Cataldo et al. 1990; Harvey et al. 1991) were employed. The plant extraction procedure for TNT is shown in Figure 2.1 while that of RDX is given in Figure 2.2. Samples were run in triplicate with appropriate internal spikes.

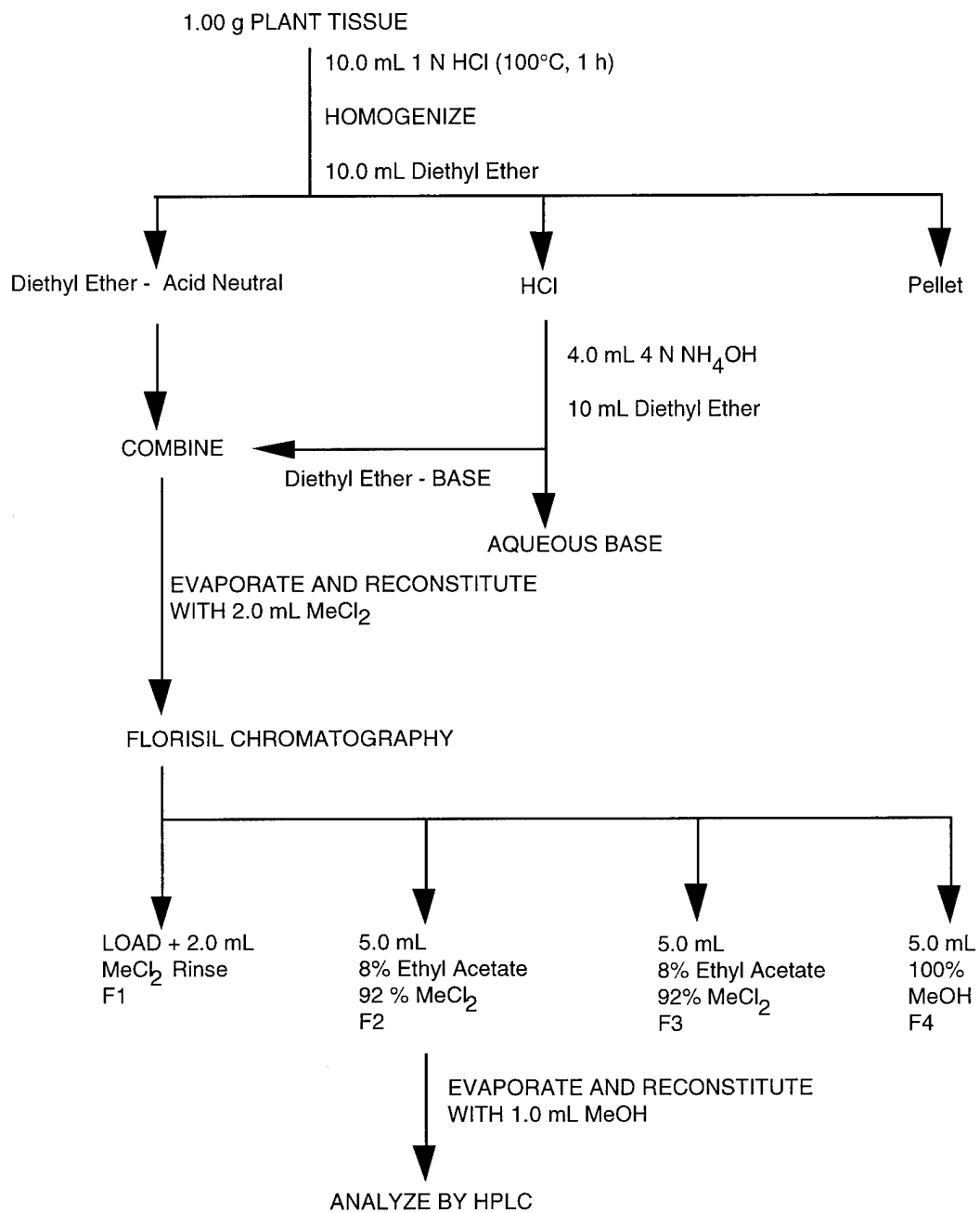


FIGURE 2.1. Flow Chart for the Analysis of TNT in Plant Tissues

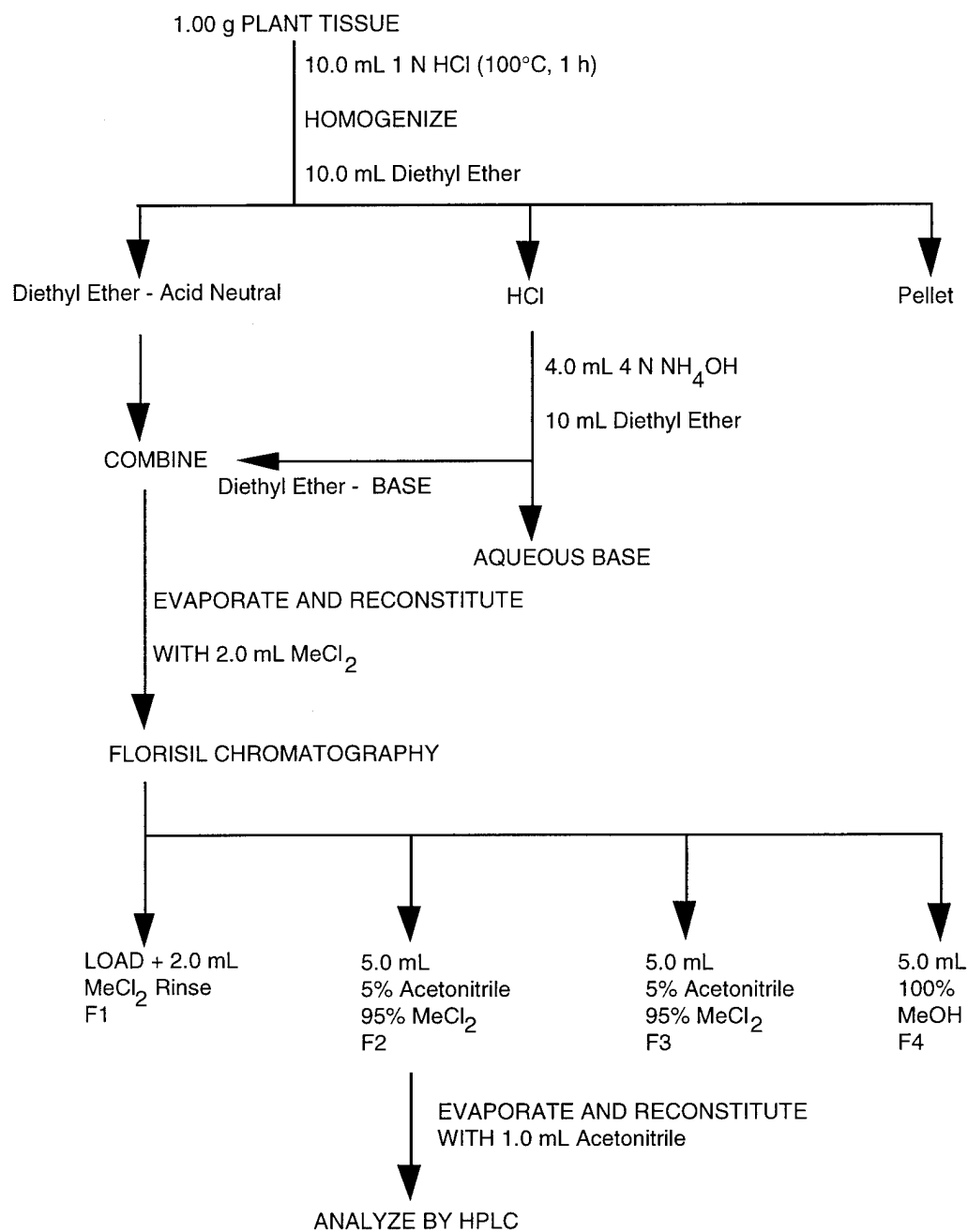


FIGURE 2.2. Flow Chart for the Analysis of RDX in Plant Tissues

3.0 RESULTS AND DISCUSSION

The overall purpose of this effort was to investigate the behavior and impacts of munitions residues released to the environment. The objective was to develop an understanding of the variations in long-term partitioning and metabolism of munitions (TNT, RDX) in tissues of various crop plants expected to be vital food chain components associated with operating or released production facilities. This study represents a logical follow-on inquiry based on the results of currently completed activities (TNT, RDX, Tetryl) sponsored by USABRDL.

Previous efforts in our laboratory have shown a significant inverse correlation between the uptake of munitions compounds by plants and the organic matter content/fertility of the amended soil the plants were grown in (Cataldo et al. 1989, 1990, 1993; Fellows et al. 1993a). We have tested a high-fertility mountain soil (Cinebar), a moderate-fertility agricultural soil (Palouse), and a low-fertility desert soil (Burbank). In all instances (differing plant species and munitions material) munitions accumulation has been the highest in those plants grown in the Burbank soil. Because the ultimate thrust of this program was an evaluation of potential risks to human food chains from munitions materials, only Burbank soil was used to grow the plants to maturity or to that stage when they would normally be harvested as agricultural products.

3.1 DISTRIBUTION OF MUNITIONS RESIDUES IN PLANT TISSUES AT MATURITY

To date, all of the plant species studied are capable of growing in soils contaminated by TNT and RDX and will accumulate these materials and metabolites within their tissues. This accumulation can be expressed both in terms of specific activity (μg munition or munition-equivalent/g dry wt. of tissue) for the individual portions of the plant, and in terms of actual uptake (μg munition or munition-equivalent/tissue or plant). The former will allow direct comparisons between tissues of different plant species on a standardized basis (g dry wt.). The latter will permit evaluation of uptake efficiency (how much of the contaminant present can the plant accumulate over a given time period) and where the plant partitions the contaminant within its various tissues on a percentage basis. Both forms of analysis will be presented in Section 3.1.

3.1.1 TNT Uptake and Distribution

Plants are not only capable of growing in soils contaminated by TNT but will also accumulate the material and its metabolites within their tissues (Palazzo and Leggett 1986; Harvey et al. 1990). In most instances, the actual TNT content of these tissues is low with the majority of the parent TNT being converted to 2- and 4-ADNT and other more polar metabolites either at the surface of the root or within the root tissue (Harvey et al. 1990; Fellows et al. 1993a; Schneider et al. 1994). The form of TNT transported within the plant appears to primarily be a polar conjugate of either TNT or the 2- and 4-ADNTs with only a small amount of parent TNT evident (Harvey et al. 1990; Fellows et al. 1993a). Because of this apparently rapid metabolism, actual uptake values of the material are difficult to assess through standard analytical techniques and mass balance studies are impossible. The use of radiolabeled TNT parent material which can be traced and quantified either as a metabolite or in an unaltered form, is critical in the determination of the actual uptake capability of the plants. In light of this, the data presented in this section will be expressed as ^{14}C -TNT-equivalents even though their actual composition may be different than that of the parent compound. Actual analytical analysis of the material found within the plant will be presented in Section 3.2.

The data in this section are presented in two separate groupings with the carrot, spinach, and alfalfa plants comprising one group (Tables 3.1 to 3.4) and the corn (Table 3.5) the other. For each of the three species in the first group, the normally consumable portion of the plant will be either the root (i.e., carrot) or the shoot (i.e. spinach and alfalfa) with the shoot being comprised of the stem and leaves. The corn is commonly divided into the ear (containing the cob and seed), and the shoot (containing the tassel, leaves, and stem) upon harvesting, and so these parts were treated separately.

All of the data presented in Tables 3.1 to 3.6 represent the munitions-amended plants. Control plants were grown in the same chambers and in separate growth chambers (chamber controls) over the same time period. In all instances, no label was found in the chamber control plants. Control plants growing in the same growth chambers as the ^{14}C -TNT-amended plants did have a background value of ^{14}C within their tissues of approximately 0.52% that of the ^{14}C -TNT plants. This was the same as had been observed in the initial study (Cataldo et al. 1989). The partitioning patterns of these control plants were again consistent with that of $^{14}\text{CO}_2$ assimilation, and these patterns were concluded to be the result of soil mineralization of the ^{14}C -TNT. This background level was subtracted.

In the initial study on the uptake of soil-amended ^{14}C -TNT by bean, wheat, and grass (Blando Brome), the Burbank soil was amended to a final concentration of 10 $\mu\text{g/g}$ dry wt. with

TNT. Soil contaminant concentration in the current study was increased to 30 µg/g dry wt. of soil. While the concentration was below levels previously shown to evoke phytotoxic responses under these growth conditions (50 µg/g dry wt., Cataldo et al. 1989), it was chosen to maximize uptake to assess potential environmental problems. In addition, because of the higher TNT concentration to be employed, and in an attempt to minimize potential phytotoxicity and reflect soils which have been contaminated for an extended period, the soil was allowed to equilibrate (without plants) for 14 days prior to seeding as opposed to the previously employed 48 h.

The initial work was expressed in terms of µg TNT-equivalents/g fr. wt. of tissue (Cataldo et al. 1989; Harvey et al. 1990). Values of TNT-equivalents found within the tissues of these plants grown from seed ranged from 59±11 µg/g fr. wt. for the roots of grass to 217±60 µg/g fr. wt. for wheat roots. Using a correction factor for conversion from fresh weight to dry weight for the same plants grown under identical conditions (0.127, Fellows et al. 1993a), the specific activity (µg/g dry wt.) in TNT-equivalents would then range from 464±86 for the grass to 1708±470 for the wheat.

These former values are approximately one-third of those observed in the present study (Table 3.1). However, given the three-fold increase in soil TNT concentration used compared to the 1989 work, this suggests that there may be a linear response to increasing contaminant soil concentrations over these sub-phytotoxic concentration ranges and would verify the initial observations.

A marked difference exists in the present study between the specific activity of the spinach roots and those of the other plants (Table 3.1). The specific activity found in these tissues is much higher than the other species tested; this could indicate a greater ability for TNT accumulation from the soil.

TABLE 3.1. Specific Uptake (µg ¹⁴C-TNT Equivalents/g dry wt.) in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 30 µg/g ¹⁴C-TNT. Data are averages (n=6) ± s.e.

Plant Species	µg ¹⁴ C-TNT Equivalents/g dry wt		
	Seed and Blossom	Shoot	Root
Carrot	^a	387.9±56	175.1±38
Spinach	24.9±10	772.5±118	4658.3±783
Alfalfa	11.2±3	86.7±22	196.3±52

^aSeeds not present.

Part of the higher ^{14}C activity in the spinach roots may have been a higher background activity (adhering contaminated soil to the root mass) than actual assimilated label. However, care was always taken to remove as much external contamination as possible during harvest for all plants. This included three successive washes in water, 80% (v/v) methanol, and 0.05 M CaCl_2 , respectively.

The total plant dry weight of the control spinach was three times smaller than those of plants growing in the TNT-amended soils (0.66 ± 0.19 versus 0.21 ± 0.06 g) (Table 3.2). This suggests that there was little evident toxicity from the TNT. Therefore, if the roots were active and relatively free of background contamination then an enhanced TNT uptake capability would be favored. This enhanced uptake would also account for the elevated specific activity found in the leaves of these spinach plants shown in Table 3.1 compared to the other two species.

Note similar significant increases in the dry weights of the carrot and alfalfa plants were observed for those plants grown in the TNT-amended soil (Table 3.2). While a similar observation was not apparent in earlier studies for other species grown in Burbank soil at lower TNT concentrations, the possible significance of this current result is discussed in Section 3.3 in terms of plant nutrition.

TABLE 3.2. Dry Weights (g) of Alfalfa, Spinach, and Carrot Plants Grown for 70 to 90 Days on Burbank Soil Alone (Control) or Amended with $30 \mu\text{g/g}$ ^{14}C -TNT (Amended). Data are averages ($n=6$) \pm s.e.

Plant Species	Dry Wt. (g)	
	Control	Amended
Carrot	0.52 ± 0.17	2.92 ± 1.81
Spinach	0.32 ± 0.08	0.66 ± 0.19
Alfalfa	2.43 ± 0.82	4.37 ± 0.67

The actual amount of label accumulated by each tissue of the carrot, spinach, and alfalfa plants and their sums expressed as μg of ^{14}C -TNT-equivalents is presented in Table 3.3. This shows both the actual contents in μg TNT-equivalents of each organ within the plant to enable direct comparison between species grown under the same conditions as well as the relative accumulation efficiency of the plant. This data is also given as percent of total label for each species in Table 3.4.

Two items are of note from Table 3.3. First, by using the total activity found in each plant and the dry wt. from Table 3.2, a ratio of activity/plant wt. can be constructed. The results of this show that the ratio for the spinach was twice that of the carrot and three times that of the alfalfa; thus, further indicating that the spinach may have a higher rate of contaminant accumulation than the other two. Secondly, the total accumulation of TNT by the plants was only a small portion of the total labeled compound originally amended to the soil. These values ranged from 1.24% for the spinach to 2.34% for the carrot. This small value indicates that even though the plants may have had some tissues with extremely high specific activities (see Table 3.1), overall the plants were not significant accumulators of the TNT or TNT-derived label from the Burbank soil.

TABLE 3.3. Whole Tissue Concentrations of ^{14}C -TNT-Equivalents in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 30 $\mu\text{g/g}$ ^{14}C -TNT. Data are averages ($n=6$) \pm s.e.

Plant Species	$\mu\text{g } ^{14}\text{C-TNT-Equivalents}$				Percent Plant Uptake of Total Amended TNT
	Seed and Blossom	Shoot	Root	Sum	
Carrot	^a	213.8 \pm 46	347.9 \pm 73	561.6 \pm 175	2.34
Spinach	11.1 \pm 5	120.8 \pm 29	106.2 \pm 29	296.5 \pm 85	1.24
Alfalfa	3.1 \pm 2	146.0 \pm 21	431.4 \pm 111	653.5 \pm 245	2.73

^a Seeds not present.

The initial TNT studies with bean, wheat, and the grass, Blando Brome, demonstrated a general tendency for the label to accumulate in the roots of the plants as opposed to the above-ground portions (Cataldo et al. 1989). When grown in Burbank soil, the beans retained 75% and wheat 80% of the label in their roots while grass retained only 49%. However in the other soils tested (Cinebar and Palouse) the grass was similar to the beans and wheat (78 and 67%, respectively). In the present study, spinach retained only 36% of the label in its roots (see Table 3.4). The roots of these plants are much smaller in mass (0.028 \pm 0.02 g dry wt.) when compared to those of the carrot (2.324 \pm 1.298 g dry wt.) or the alfalfa (2.133 \pm 0.339 g dry wt.). With these differences in total mass, the lower percentage is not as significant. This again suggests a much higher rate of accumulation by the roots and apparent transport of the labeled compound to the shoot by the spinach.

Both the carrot and alfalfa plants shown in Table 3.4 as well as the corn shown in Table

3.5 demonstrated a higher percentage of label retained in the roots (62, 73, and 83%, respectively). These were comparable to a previous study by Cataldo et al. (1989). Cataldo and his associates suggested that the TNT may be either bound to the roots of these species like the structurally similar dinitroaniline herbicides, or the majority of the TNT might be quickly metabolized in the root to a nontransportable form (Cataldo et al. 1989; Fellows et al. 1993b).

In the case of the carrot, is most of the TNT deposited in the outer epidermis or is it distributed throughout the root? This question is important because some soil contaminants, notably transuranic radionuclides, can be removed from root tuber vegetables (e.g., carrots, beets, and potatoes) simply by peeling the root to remove the epidermal layers. Further, if the TNT was restricted to the epidermis, this would be additional evidence that much of the metabolic products observed in the shoot tissue might have been produced upon uptake and transport at the roots' surface. To address this question, cross sections of a root from a carrot grown in the ^{14}C -TNT-amended soil were taken. The slices were divided into two equal portions and one-half weighed, oxidized and counted. The remaining half was further dissected into the epidermal and cortical areas of the outer portion of the root and the inner vascular cambium area (Esau 1965). These tissues were also weighed, oxidized, and counted.

The outer portions of the root contained $55.3 \pm 4\%$ of the total label for that half cross section while the inner portion contained $44.7 \pm 6\%$. The undissected halves of the slices contained an equal amount of label on a weight basis when compared to the summed activity of the others. This indicated that the TNT is distributed evenly across the root and removal of the epidermal layers by peeling would not significantly alter the potential for food chain transfer.

TABLE 3.4. Percent Distribution of Radiolabel in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with $30 \mu\text{g/g}$ ^{14}C -TNT. Data are averages ($n=6$) \pm s.e.

Plant Species	%Total Label		
	Seed and Blossom	Shoot	Root
Carrot	a	38.3 ± 4.3	61.7 ± 4.3
Spinach	3.9 ± 2.3	60.1 ± 2.9	36.0 ± 2.6
Alfalfa	0.7 ± 0.5	25.9 ± 8.2	73.4 ± 5.0

^aSeeds not present.

The summary corn data is presented in Table 3.5. Of all of the tissues, the roots again contained the highest specific activity, actual total TNT-equivalents, and highest percent of label distribution. This is consistent with the previous observations. Further, while the specific activity ($\mu\text{g TNT-equivalents/g dry wt.}$) of the corn roots is higher than either the carrot or alfalfa, it is still considerably less than that of the spinach (Table 3.1).

The next highest amount of label was found in the leaves of the corn plant which have a high total volume of transpiration from the large leaf surface area (Fitter and Hay 1987). The loss of water from the leaves may promote a higher concentration of the xylem transported polar TNT-conjugate within the tissues because $^{14}\text{C-TNT}$ was not observed to volatilize from plant shoots (Cataldo et al. 1989).

Unlike the other three species tested in this study, the total plant dry wt. of the corn did not respond to the TNT amendment. No indication of increased dry matter content of the exposed plants existed. Also, no significant toxicity was evident. A possible explanation may lie in the pot-to-plant size ratio. The other species were much smaller in stature and total dry wt. than the corn (approximately five times less for alfalfa, the largest of the three). These plants were grown in 1-kg pots compared to the 4-kg pots of the corn. The pot size selection for the corn was constrained both by growth chamber space as well as handling considerations during the experiment. The smaller pot-to-plant ratio may have promoted root crowding or so-called "pot-binding" which may have restricted or hidden actual growth differences.

The low allocation of the label (and hence TNT and TNT-metabolites) to the reproductive parts of the plant (tassels, ear) suggests that these organs would not be a source of continued food chain contamination. Analysis of the ear itself for label partitioning showed an even distribution between the cob and seed ($46\pm6\%$ vs. $54\pm3\%$, respectively). This further suggests that the directly human consumable portion of the plant (the seed) would be a low source of TNT-contaminants.

TABLE 3.5. Specific Uptake ($\mu\text{g } ^{14}\text{C-TNT-Equivalents/g dry wt.}$), Total Tissue Content ($\mu\text{g } ^{14}\text{C-TNT-Equivalents}$), and Percent Distribution of Radiolabel in Corn Plants Grown for 100 to 120 Days on Burbank Soil Amended with 30 $\mu\text{g/g}$ TNT. Data are averages ($n=6$) \pm s.e.

Sample	Plant Organ					Sum
	Tassel	Leaves	Stem	Ear	Root	
$\mu\text{g/g dry wt. TNT-Equivalents}$	2.2 \pm 1.0	13.8 \pm 4.2	2.6 \pm 0.4	1.0 \pm 0.4	576.9 \pm 80.0	
Total Tissue $\mu\text{g TNT-Equivalents}$	1.1 \pm 0.6	189.9 \pm 55.0	31.2 \pm 9.0	5.7 \pm 1.0	1338.9 \pm 353.0	1565.1 \pm 297
Percent Distribution	0.08 \pm 0.03	17.72 \pm 5.1	2.21 \pm 0.7	0.29 \pm 0.1	83.71 \pm 5.8	100.0
Control Plant Dry Wt.	0.6 \pm 0.5	15.5 \pm 4.8	12.4 \pm 3.5	4.9 \pm 3.13	2.4 \pm 1.2	35.1 \pm 9.2
TNT-Exposed Plant Dry Wt.	0.5 \pm 0.2	12.4 \pm 3.3	12.0 \pm 2.7	3.37 \pm 1.7	2.6 \pm 0.5	31.1 \pm 4.2

3.1.2 RDX Uptake and Distribution

Few references in the literature deal with the uptake of RDX by higher plants. These studies, however, have shown that RDX-derived metabolites can accumulate in all of the plant's tissues (Cataldo et al. 1990; Harvey et al. 1991) and that the concentration in these tissues can rise with increases in contaminant soil concentration (Banwart et al. 1991). Harvey et al. (1991) have shown that while RDX is more resistant to metabolism than TNT, some polar metabolites may be present. As for TNT, the use of radiolabeled RDX parent material which can be traced and quantified either as a metabolite or in an unaltered form is critical. Therefore, the data presented in this section will also be expressed as $^{14}\text{C-RDX-equivalents}$ even though their actual composition may be different than that of the parent compound. Chemical analysis of the material found within the plant is presented in Section 3.2.

The data below are also presented in two separate groupings with the carrot, spinach, and alfalfa plants comprising one group in Tables 3.6 to 3.9, and the corn in Table 3.10, the other. The data for each species are again subdivided according to the reasoning described in Section 3.1.1.

The final RDX soil concentration selected for the present study was 15 µg/g dry wt. soil. This was below levels previously shown to evoke phytotoxic responses under these growth conditions (25 µg/g, Cataldo et al. 1990) but was higher than the previous work so as to optimize the assessment of environmental risk.

All of the data presented in Tables 3.6 to 3.10 represent the munitions-amended plants. Control plants were also grown in the same chambers and in separate growth chambers (chamber controls) during the same period. In all instances, no label was detected in the chamber control plants. Control plants growing in the same growth chambers as the ^{14}C -RDX - amended plants did have a ^{14}C background value of approximately 0.05% that of the ^{14}C -RDX plants. This was the same percentage that was observed in the initial study (Cataldo et al. 1990). The partitioning patterns of these new control plants were consistent with that of $^{14}\text{CO}_2$ assimilation. These patterns were the result of soil mineralization of the RDX to $^{14}\text{CO}_2$. This background level, however, was 10 times less than that observed for the TNT, again indicating a lower rate of soil mineralization. These background values were also subtracted from the data.

The data from the initial study was expressed in terms of µg RDX-equivalents/g fr. wt. of tissue (Cataldo et al. 1990; Harvey et al. 1991). Values of RDX-equivalents found within the roots of these plants grown from seed ranged from 28 ± 6 µg/g fr. wt. for the roots of grass to 75 ± 11 µg/g fr. wt. for bean roots. Using a correction factor for conversion from fresh weight to dry weight for roots of plants grown under identical conditions (0.127, Fellows et al. 1993a), the specific activity (µg/g dry wt.) in RDX-equivalents would then range from 220 ± 47 for the grass to 590 ± 86 for the bean. These values are comparable to the ranges shown in Table 3.6 of 313.5 ± 32 to 528.9 ± 119 µg RDX-equivalents/g dry wt. for the alfalfa and spinach roots, respectively.

The activity values between the different tissues (root, shoot, and seed) of spinach and alfalfa do not vary significantly suggesting that all of these tissues possess a similar potential for RDX accumulation. An exception to this may be the carrot shoot where significantly higher activity was found (>4200 µg RDX-equivalents/g dry wt., Table 3.6). While there was an apparent, although not statistically significant, reduction in the dry weights of the roots of carrots grown in the RDX soil (Table 3.7), this would not account for the much greater transport (partitioning) of the label to the shoots of these plants also evident in Tables 3.8 and 3.9. Therefore, the shoots of this species may have an enhanced potential for RDX accumulation.

TABLE 3.6. Specific Uptake ($\mu\text{g } ^{14}\text{C-RDX-Equivalents/g dry wt.}$) in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with $15 \mu\text{g/g RDX}$. Data are averages ($n=6$) \pm s.e.

Plant Species	$\mu\text{g } ^{14}\text{C-RDX-Equivalents/g dry wt.}$		
	Seed and Blossom	Shoot	Root
Carrot	^a	4294.3 \pm 751	468.7 \pm 123
Spinach	499.3 \pm 93	832.2 \pm 178	528.9 \pm 119
Alfalfa	377.2 \pm 51	429.9 \pm 102	313.5 \pm 32

^aSeeds not present.

No significant differences were present in the dry weights of the three species grown in either the control soil or in the RDX-amended soil (Table 3.7). Similar observations were made for bean and wheat in a previous study by Cataldo et al. (1989). This lack of significant differences further indicates that at this concentration ($15 \mu\text{g/g}$) the RDX did not exert a phytotoxic effect (i.e., reduction of dry matter accumulation) in these plants. Further, it points to a further variation between RDX and TNT where the latter may have slightly promoted growth (see Section 3.3)

TABLE 3.7. Dry Weights (g) of Alfalfa, Spinach, and Carrot Plants Grown for 70 to 90 Days on Burbank Soil Alone (Control) or Amended with $15 \mu\text{g/g } ^{14}\text{C-RDX}$ (Amended). Data are averages ($n=6$) \pm s.e.

Plant Species	Dry Wt. (g)	
	Control	Amended
Carrot	5.20 \pm 1.58	3.92 \pm 3.54
Spinach	2.47 \pm 0.56	2.49 \pm 1.25
Alfalfa	4.93 \pm 1.38	4.98 \pm 1.64

Tables 3.8 and 3.9 show that the majority of the RDX extracted by the plant from the soil was partitioned into the shoot with only a small portion retained by the root. This pattern has been reported previously (Cataldo 1990) in wheat and bean. The partitioning percentages for the current study ranged from $>98\%$ of the total radioactivity for the spinach to almost 84% for the alfalfa (Table 3.9). Significantly high amounts ($>200 \mu\text{g/g dry wt}$) were present in the flowers and seed of these plants (Table 3.8). Spinach has few stomata and, therefore, little or no

transpiration from these structures. This suggests that a portion of the RDX or RDX metabolites would have to be phloem mobile which would enhance its plant partitioning potential.

Table 3.8 indicates that these plants were able to accumulate over 15 to 37% of the total label present in the pot over the growing season. Previously, Cataldo (1990) reported that 11 to 55% of the soil-amended RDX was removed by bush bean, and 10 to 40% was removed in a single harvest of wheat and Blando Brome. This further indicates that plants are capable of accumulating significant portions of the RDX contaminant from the soil. This property could prove a useful component in a managed bioremediation scheme of near-surface RDX munition-contaminated soil.

TABLE 3.8. Whole Tissue Concentrations of ^{14}C -RDX-Equivalents of Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 15 $\mu\text{g/g}$ ^{14}C -RDX. Data are averages ($n=6$) \pm s.e.

Plant Species	$\mu\text{g } ^{14}\text{C-RDX Equivalents}$			Percent Plant Uptake of Total Amended RDX
	Seed and Blossom	Shoot	Root	
Carrot	^a	4160.4 \pm 861	372.2 \pm 99	37.3 \pm 8
Spinach	658.6 \pm 302	1183.2 \pm 235	30.8 \pm 15	15.4 \pm 5
Alfalfa	220.9 \pm 71	1533.3 \pm 259	351.1 \pm 117	16.6 \pm 4

^aSeeds not present.

To determine if there was a partitioning of the RDX in the epidermis of the carrot, a cross-section of a ^{14}C -RDX grown root was taken and processed like those described for the TNT experiment (Section 3.1.1). These tissues were also oxidized and counted. The outer portions of the RDX root contained 59.1 \pm 5% of the total label while the inner portion contained 40.9 \pm 7%. This indicated that the RDX, like the TNT, is evenly distributed within the root. Removal of the epidermal layers by peeling would not significantly alter the potential for food chain transfer.

TABLE 3.9. Percent Distribution of Radiolabel in Carrot, Spinach, and Alfalfa Plants Grown for 70 to 90 Days on Burbank Soil Amended with 15 $\mu\text{g/g}$ ^{14}C -RDX. Data are averages ($n=6$) \pm s.e.

Plant Species	%Total Label		
	Seed and Blossom	Shoot	Root
Carrot	a	91.6 \pm 2.6	8.4 \pm 2.6
Spinach	32.7 \pm 7.6	65.66 \pm 7.4	1.6 \pm 0.6
Alfalfa	10.8 \pm 3.7	73.3 \pm 5.3	15.9 \pm 3.0

^aSeeds not present.

The summary RDX corn data are presented in Table 3.10. Consistent with the other plant species tested, the roots contained only a small part (<6%) of the total label (RDX-equivalents). The leaves were the tissue with the highest specific activity. This is attributed to the apparent mobility of RDX around the plant (Cataldo et al. 1990), the large surface area of the leaves with the accompanying high phloem transport activity, and transpiration rate associated with this tissue (Edwards and Walker 1983), and the fact that RDX is not volatilized from the plants.

No significant differences were noted in the dry wt. of the plants grown in the control soils and those grown in soil amended with RDX. Within the plant, a slight tendency for reduced stem size was found in the RDX plants (Table 3.10) but this was not significant.

Contrary to the TNT plants, the reproductive parts of the plant (tassels, ear) received a significant allocation of the label. While the labeled material observed was RDX-equivalents, the plant still contains significant amounts of the parent material (Harvey et al. 1991) and may become a secondary source of contamination to the environment. This is particularly true for the pollen in the tassels.

Analysis of the ear for label partitioning as done for the TNT again showed an even distribution between the cob and seed (58 \pm 9% vs. 42 \pm 6%, respectively). The specific activity of the ear (112.9 \pm 17 μg RDX-equivalents/g dry wt.) suggests that both the seed and the cob, although containing a only small portion of the total plant radiolabel (5.3%), may prove a potential source of food chain contamination.

TABLE 3.10. Specific Uptake ($\mu\text{g } ^{14}\text{C-RDX-Equivalents/g dry wt.}$), Total Tissue Content ($^{14}\text{C-RDX-Equivalents}$), and Percent Distribution of Label in Corn Plants Grown for 100 to 120 Days on Burbank Soil Amended with $15 \mu\text{g/g RDX}$. Data are averages ($n=6$) \pm s.e.

Sample	Plant Organ					Sum
	Tassel	Leaves	Stem	Ear	Root	
$\mu\text{g/g dry wt. RDX-Equivalents}$	326.3 \pm 25	633.5 \pm 83	212.8 \pm 34	112.9 \pm 17	359.7 \pm 23	
Total Tissue $\mu\text{g RDX-Equivalents}$	584.1 \pm 161	7147.6 \pm 1151	745.6 \pm 72	312.1 \pm 149	480.9 \pm 78	9455.1 \pm 2256
Percent Distribution	6.1 \pm 1.1	75.4 \pm 1.4	8.1 \pm 1.2	5.3 \pm 1.7	5.1 \pm 0.5	100.0
Control Plant Dry Wt.	1.3 \pm 0.6	11.9 \pm 5.1	8.4 \pm 3.9	3.6 \pm 2.3	1.9 \pm 1.1	27.2 \pm 11.0
RDX-Exposed Plant Dry Wt.	1.8 \pm 0.7	11.3 \pm 1.4	3.6 \pm 0.6	3.6 \pm 1.4	1.3 \pm 0.3	21.7 \pm 1.1

3.2 CHEMICAL ANALYSIS OF INDIVIDUAL TISSUES FROM SOIL-GROWN PLANTS

Both the TNT and the RDX undergo metabolic transformations when assimilated by a higher plant. Of the two, the TNT appears to be more quickly metabolized (Harvey et al. 1990, 1991, 1992). Harvey et al. (1990) have shown that TNT is quickly reduced to its 2- and 4-ADNT isomers in the plant. Cataldo et al. (1989) reported that in bean plants grown for 60 days in $10 \mu\text{g/g } ^{14}\text{C-TNT}$ -amended Burbank soil only a small amount of the TNT-derived radioactivity was associated with TNT (1 to 2%) and the ADNT isomers (~20%) while the remainder was associated with insoluble tissue residues.

The fate of RDX in bean plants grown under similar exposure conditions was different. Only 20% of the radioactivity present was found as parent RDX in the roots and stems of these plants. However greater than 50% of the radioactivity was identified to be RDX in the leaves and seed. The remainder of the radiolabel was associated with unidentified polar metabolites or insoluble plant fractions (Cataldo et al. 1990; Harvey et al. 1991).

In the present study the corn and alfalfa were grown for a longer period (up to 120 days) in soils amended at a slightly higher concentration. Two types of tissues, the leaves of corn and the shoot of alfalfa, were selected for analysis. This decision was based both on their higher

radiolabel contents as well as their increased potential as starting points of food chain interactions (silage and hay).

Both the TNT and RDX are soluble in nonpolar conditions. In the present analytical scheme the parent compounds of RDX and TNT and the 2- and 4- ADNTs were found to elute in the F2 fraction. The remaining fractions contained polar-extractable metabolites while the pellet contained the insoluble material. Results of the analyses expressed as percent of total radioactivity for the different extracted fractions and insoluble pellets are given in Tables 3.11 and 3.12. Identification of the actual material in the F2 fraction was confirmed using HPLC techniques (Harvey et al. 1990, 1991).

The data indicated that the percentage of label found in the F2 fraction of the RDX plants was higher than that of the TNT tissues in the alfalfa and corn (6.62 vs. 23.45% and 2.64 vs. 8.65%, respectively) (Tables 3.11 and 3.12). The highest F2 values were found in the RDX alfalfa tissue (23.45%, Table 3.11). HPLC analysis showed a concentration of 184 µg RDX/g for this fraction (data not shown). This is high but is comparable to the 114 to 282 µg RDX/g found in wheat and grass shoots, respectively (Cataldo et al. 1990). Note that the insoluble pellet contained 67% of the total radioactivity and so the total amount in the F2 was significantly higher by comparison (almost half of the extractable activity).

TABLE 3.11. Percentage of Total Radioactivity in the Various Chemical Fractions of Alfalfa Stems from Plants Grown to Maturity (90 to 120 days) in ¹⁴C-TNT (30 µg/g dry wt. soil) or ¹⁴C-RDX (15 µg/g dry wt. soil) Amended Burbank Soil.

Fraction	Amendment	
	TNT	RDX
HCl	21.26	49.86
Ether acid-neutral	a	a
Aqueous base	23.32	44.19
Ether base	a	a
F1	5.43	0.64
F2	6.62	23.45
F3	2.68	1.19
F4	8.37	2.66
Sorbent	0.32	0.31
Pellet	42.84	67.02

^a Ether fractions were combined.

TABLE 3.12. Percentage of Total Radioactivity in the Various Chemical Fractions of Corn leaves from Plants Grown to Maturity (90 to 120 days) in ^{14}C -TNT (30 $\mu\text{g/g}$ dry wt soil) or ^{14}C -RDX (15 $\mu\text{g/g}$ dry wt. soil) Amended Burbank Soil

Fraction	Amendment	
	TNT	RDX
HCl	14.08	29.24
Ether acid-neutral	9.53	8.19
Aqueous base	12.13	22.17
Ether base	4.21	11.98
F1	1.73	4.20
F2	2.64	8.65
F3	4.62	3.25
F4	12.43	13.12
Sorbent	0.15	0.15
Pellet	70.4	46.65

HPLC analysis of the corn leaf F2 fraction showed a lower concentration of almost 19 μg RDX/g. While lower than that of alfalfa, the F2 fraction of the corn leaf tissue contained only 8.65% of the soluble fraction with the majority of the soluble radioactivity found in the polar metabolites. Lower F2 percentages were also found in both TNT plants. However, in this case, HPLC analysis failed to identify (<0.01 $\mu\text{g/g}$) TNT or the 2- and 4-ADNT metabolites in the tissues. This suggests that the radiolabel found in the corn leaf is almost entirely insoluble, bound, or polar-conjugates/metabolites of the original TNT.

For TNT metabolites, the actual environmental impact is unknown at this time but this information confirms what has been recently reported by Schneider et al. (1994) when they were unable to detect TNT or ADNTs in corn grown on TNT-contaminated soil at a former munitions plant. Possible food chain effects from such an entry point are unknown.

For RDX, the more immediate concern is that substantial amounts of the parent RDX material are present in the forage tissues of a significant agricultural crop. While the toxic effects of direct parent compound RDX dosage to herbivores has been reported (Merck 1983), no information is available on potential impacts from these polar metabolites found in primary herbivore feedstock, such as alfalfa, growing on contaminated sites. However, one of the reported metabolites in bacteria, hydrazine, is a known mutagen (McCormick et al. 1981).

3.3 DETERMINATION OF ALTERATIONS IN PLANT PARTITIONING WITH VARIATIONS IN PLANT NUTRITIONAL STATUS

The Burbank soil selected for the partitioning studies (Section 3.1) has consistently exhibited the highest rates of plant xenobiotic uptake (Cataldo et al. 1989, 1990, 1993; Fellows et al. 1993a). This has correlated with the soil's relatively low CEC of 5.5 meq/100 g and low organic matter content of 0.5%. The principle plant nutrient missing from this soil type when compared to the others tested is nitrogen (N).

Plants will preferentially extract nitrogen from the soil in the form of NO_3 and subsequently reduce this to NH_3 (Dennis and Turpin 1990). Legumes, such as alfalfa, while preferring NO_3 as a nitrogen source, also possess the capability of meeting their nitrogen requirements through a symbiotic relationship with a soil microbe, the *Rhizobia* spp. The *Rhizobia* are capable of atmospheric nitrogen fixation and provide this fixed nitrogen form to the plants in exchange for a carbon source and a protected environment (a root nodule). If soil nitrogen is limited, the plant may seek alternate nitrogen forms which could be reduced and metabolized. Because TNT has been shown to rapidly convert to such reduced forms as 2- and 4-ADNTs in soil (Cataldo et al. 1989; Harvey et al. 1990), TNT could serve as an alternate or supplemental nitrogen source. While there were no significant increases in plant fresh weight in the previous studies with plants growing in TNT-amended Burbank soil (Cataldo 1989) a slight increase was subsequently observed in the dry weights of plants grown in Tetryl-amended Burbank soil (Fellows et al. 1993a). The chemical similarity of TNT and Tetryl and the results of the experiments described in Section 3.1.1 indicated that this possibility needed to be addressed.

Burbank soil, as is common to many of the soils of Eastern Washington (D. Bezdicsek, 1988, Washington State University, personal communication), is very low in native *Rhizobia* and requires supplemental inoculation to initiate root nodulation. Thus, it was possible to add either *Rhizobia* and TNT, nitrogen fertilizer and TNT, or TNT alone to control all three potential nitrogen sources.

As an experimental approach, alfalfa plants were grown in ^{14}C -TNT-amended Burbank soil (at a final concentration of 15 $\mu\text{g/g}$ dry wt. to more closely reflect those used for the original studies [Cataldo 1989]) and control nonamended Burbank soil in the same growth chambers. The plants were grown for 45 days as described in Section 2.0. In addition to the presence or absence of TNT, the treatments included either the Burbank soil alone, the Burbank soil inoculated with a suitable amount (1×10^6 propagules/g dry wt. of soil) of commercial *Rhizobium* inoculum (*Rhizobium meliloti*, Nitagin, Liphatech, Inc. Milwaukee, WI, Lot no. AB20), or the Burbank soil provided with a supplemental nitrogen fertilizer at a rate of 30 mL/pot

(0.005% w/v of Peters Professional Water Soluble Fertilizer, 20-20-20; W.R. Grace and Co., Fogelsville, PA) every 10 to 14 days. The results expressed as either $\mu\text{g/g}$ dry wt. of ^{14}C -TNT-equivalents, total μg of ^{14}C -TNT-equivalents per tissue, percent distribution of assimilated label, or percent of total amended label are given in Table 3.13.

The alfalfa plants were still vegetative when harvested and although small in stature (10- to 25-cm tall) were just beginning to show enhanced growth. The specific activity is higher than in the earlier experiment for 120-day-old plants discussed in Section 3.1 and may be reflective of their younger developmental stage. This is particularly evident in those plants grown on the TNT-amended plain Burbank soil where their root activity exceeded 14,000 μg TNT-equivalents/g dry wt. Although all precautions were taken to remove contaminating soil from the root, this data must be taken with caution because the average root dry weight was 0.02 g and the total activity found in these organs averaged 241 μg ^{14}C -TNT-equivalents compared to the 877 and 927 μg ^{14}C -TNT-equivalents for the *Rhizobia* and fertilizer plants, respectively.

TABLE 3.13. Specific Activity (μg ^{14}C -TNT equivalents/g dry wt. tissue), Total Tissue Contents (μg ^{14}C -TNT Equivalents), and Percent Label Distribution in Alfalfa Grown under Differing Nitrogen Treatments. Plants were grown for 45 days in Burbank soil amended with 15 $\mu\text{g/g}$ ^{14}C -TNT. Data are averages ($n=5$) \pm s.e.

Sample	Plain Soil		<i>Rhizobia</i>		Fertilizer	
	Shoot	Root	Shoot	Root	Shoot	Root
$\mu\text{g/g}$ dry wt. TNT Equivalents	3062.0 \pm 998	14389.0 \pm 2091	485.6 \pm 117	5047.0 \pm 1058	428.8 \pm 101	4805.6 \pm 1159
Total Tissue μg TNT Equivalents	80.9 \pm 28	241.0 \pm 37	144.1 \pm 30	877.7 \pm 123	167.3 \pm 35	927.3 \pm 181
Percent Distribution	24.1 \pm 4.2	75.9 \pm 4.3	13.9 \pm 1.4	86.1 \pm 1.4	15.5 \pm 2.3	84.5 \pm 2.3
Percent of Total Amended TNT Accumulated by Plant	2.68 \pm 0.48		8.52 \pm 1.25		9.12 \pm 1.69	

Given the dry weight differences between the plants and the nitrogen treatments (Table 3.14) both nutritional stress (deprivation) and munitions toxicity may have occurred. This was particularly possible given the high tissue content and specific activity values observed ($>14,000$ $\mu\text{g/g}$ dry wt.) in the plain soil plants. the plant total dry matter for the TNT plants was significantly reduced when compared to the controls. This observation differs from that found in Section 3.1.1 where higher concentrations of TNT were employed.

This paradox, however, may be related to the actual state of the TNT in the soil when the plants were seeded. In the case of the present study and in those studies conducted earlier (Cataldo 1989), the plants were seeded within 48 h of the TNT amendment while in the study in Section 3.1.1, a 14-day equilibration period was employed (precisely because of the higher concentration and the desire to reduce phytotoxic shock to the plants). The potential exists that during the longer equilibration period the TNT may have stimulated the natural soil microbial community (which was later associated with the plant roots) to promote a transformation (metabolism or complexation) of the TNT into a more readily accessible form thus facilitating a greater TNT (N) uptake and plant growth.

No TNT related toxicity (reduction in dry wt.) was apparent in the larger plants provided with a supplemental nitrogen source (Table 3.14). The added TNT-nitrogen in fact appears to have promoted dry matter accumulation. This observation complimented the similar data reported in Section 3.1.1. As regards the paradox of the plain soil, these supplemental nitrogen treatments may have also stimulated the same soil microbial community and hence the added nitrogen (TNT) uptake and plant growth.

No significant differences were evident between the supplemental nitrogen treatments (Rhizobia and fertilizer) in either plant dry weight, tissue specific activity, total tissue contents, or label distribution patterns. Therefore, while some circumstantial evidence exists for a TNT-stimulation of growth in situations where the nitrogen content of the soil may be limited, definitive proof was not obtained. Additional studies both in soils seeded within a short time after amendment, and after a long (>2 weeks) equilibration period, where the plants would be grown to the reproductive and mature stage may be needed to effectively resolve the question.

TABLE 3.14. Dry Weight (g) of Alfalfa Plants Grown for 45 days in Burbank Soil Amended With 15 µg/g ¹⁴C-TNT and Provided Different Nitrogen Treatments. Data are averages (n=5) ± s.e.

Treatment	Dry Wt. (g)	
	Control	TNT-Amended
Plain Soil	0.07±0.02	0.04±0.01
Rhizobia	0.33±0.02	0.66±0.18
Fertilizer	0.29±0.08	0.51±0.09

4.0 SUMMARY AND CONCLUSIONS

The goals of this effort were to confirm and expand data related to the behavior and impacts of munitions residues upon human food chain components potentially associated with production or testing facilities. This study was based on the results of recently completed studies (TNT, RDX) sponsored by USABRDL. The objective was to assess relative accumulation/partitioning and chemical form(s) of munitions within key crop plants so that prior USABRDL-sponsored research with TNT and RDX might be enhanced and further validated.

4.1 DISTRIBUTION OF MUNITIONS RESIDUES IN PLANT TISSUES AT MATURITY

4.1.1 TNT Uptake and Distribution

All four of the plant species used in this study accumulated the ^{14}C -TNT-derived label and distributed portions of it throughout the entire plant when grown from seed in the ^{14}C -TNT-amended soil. The study employed TNT concentrations of $30\text{ }\mu\text{g }^{14}\text{C}\text{-TNT/g dry wt. of soil}$ while the earlier work was performed at $10\text{ }\mu\text{g }^{14}\text{C}\text{-TNT/g dry wt. of soil}$. A correlation appeared between the TNT soil concentration and the plant tissue specific activities (expressed as $\mu\text{g TNT-equivalents/g dry wt.}$) at least at these concentration levels. The specific activities observed in the study were approximately three times those observed in the the earlier TNT work (Cataldo et al. 1989). A corresponding three-fold increase in the total uptake ($\mu\text{g TNT-equivalents}$) was also observed.

The carrot, alfalfa, and corn plants demonstrated a higher percentage of label retained in the roots (62, 73, and 83%, respectively). These were comparable to the previous study for wheat, grass, and bean (Cataldo et al. 1989). The spinach contained less activity in its root (36%) but also contained the highest TNT specific activity observed ($>4600\text{ }\mu\text{g TNT-equivalents/g dry wt.}$). The spinach root mass was significantly smaller than the other species tested (0.02 g vs. up to 2.6 g for the corn), and the specific activity values may have been affected. However, the ratio of the total label found within the plant versus plant total dry wt. for the spinach was twice that of the carrot and three times that of the alfalfa indicating that the spinach may have a higher rate of contaminant accumulation.

The total accumulation of TNT by the plants was only a small portion of the actual labeled compound originally amended to each pot. These accumulation values ranged from 1.24% for the spinach to 2.34% for the carrot. This small a value indicates that even though the plants may have had some tissues with extremely high specific activities, plants were not significant

accumulators of the TNT or TNT-derived label from the Burbank soil.

To date, of those species tested, the carrot was the only one where the primary consumable portion, the root, was in direct contact with the contaminated soil over the life of the plant. The outer portions of the root contained $55.3 \pm 4\%$ of the total label while the inner portion contained $44.7 \pm 6\%$. This indicated that the TNT is distributed evenly across the root and removal of the epidermal layers by peeling would not significantly alter the potential for food chain transfer.

In the corn plants, the roots contained the highest specific activity, actual total TNT-equivalents, and highest percent of label distribution. The next highest amount of label was found in the leaves. These tissues have a large surface area and a high volume of transpiration (Fitter and Hay 1987). Because ^{14}C -TNT has not been observed to volatilize from plant shoots (Cataldo et al. 1989), it was apparent that xylem transported polar TNT-conjugates (Fellows et al. 1993b) accumulated within these tissues. These tissues comprise a large portion of the dry matter chopped and stored to make silage, a food source for dairy and beef cattle, and could therefore become a potential source of food chain contamination.

The low allocation of the label (and hence TNT and TNT metabolites) to the reproductive parts of the plant (tassels, ear) suggests that these organs would not be a source of continued environmental food chain contamination even with the uniform distribution observed between the cob and seed ($46 \pm 6\%$ vs. $54 \pm 3\%$, respectively).

4.1.2 RDX Uptake and Distribution

The specific uptake values for the spinach and alfalfa were comparable to those previously reported for wheat and bean (314 to 590 μg RDX-equivalents/g dry wt. respectively) (Cataldo et al. 1990 and 1993). However, in the carrot shoot, the specific activity exceeded 4200 μg RDX-equivalents/g dry wt. with an accompanying tissue concentration of over 4000 μg . These carrot plants accumulated over 37% of the total activity amended to their pots, more than twice that of the other two species tested. Therefore, this species may have an enhanced potential for RDX accumulation.

The partitioning of the assimilated label within the root of the carrot was also studied in the RDX plants. When analyzed, the outer portions of the RDX root contained $59.1 \pm 5\%$ of the total label while the inner portion contained $40.9 \pm 7\%$. This indicated that the RDX is evenly distributed within the root.

Consistent with the other plant species tested, the roots of corn contained only a small part (<6%) of the total label (RDX-equivalents) and the leaves were the tissue with the highest specific activity and total amount of RDX-derived label. Contrary to the TNT plants there was significant portion of the label allocated to the reproductive parts of the corn plant (tassels, ear). Given the report by Harvey et al. (1991) and Cataldo et al. (1993) that much of the soil-accumulated RDX remains as the parent compound in plant tissues these dispersible plant parts may become a secondary source of contamination to the environment.

4.2 CHEMICAL ANALYSIS OF INDIVIDUAL TISSUES FROM SOIL-GROWN PLANTS

TNT and RDX assimilated by higher plants growing in contaminated soil may have differing metabolic fates. Cataldo et al. (1989) reported that after 60 days only a small amount of the TNT-derived radioactivity in bean plants was associated with TNT (1 to 2%) and ADNT isomers (~20%) while the remainder was associated with insoluble tissue residues. For RDX, ~20% of the radioactivity present was found as parent RDX in the roots and stems of these plants but in the leaves and seed this rose to 50% with the remainder associated with unidentified polar metabolites or insolubles (Cataldo et al. 1990; Harvey et al. 1991).

The leaves of corn and the shoot of alfalfa were selected for analysis in the present study based both on their radiolabel contents and their increased potential as starting points of food chain interactions (silage and hay). Only 2 to 6% of the total label was found in the F2 fractions of the TNT-grown plants. These results are generally similar in the overall pattern to those previously reported (Cataldo et al. 1989; Harvey et al. 1990). HPLC analysis of the F2 fraction, however, indicated no identifiable TNT or ADNT isomers present.

The patterns for the RDX plants were also similar to those observed previously in bean, wheat, and grass. There was higher activity in the F2 fraction of these plants and the HPLC analysis identified the parent RDX compound at significant levels in the shoot of alfalfa (>180 µg/g) and corn (>18 µg/g). Substantial amounts of the parent RDX material were therefore present in forage tissues of the mature plants.

4.3 DETERMINATION OF ALTERATIONS IN PLANT PARTITIONING WITH VARIATIONS IN PLANT NUTRITIONAL STATUS

Plants grown in the Burbank soil consistently exhibited higher rates of uptake from the soil of amended munitions material. This was correlated with the soil's relatively low CEC of 5.5 meq/100g and low organic matter content of 0.5% (Cataldo 1993). The principle plant nutrient missing from this soil type when compared to the others tested is nitrogen.

Alfalfa plants were grown in ^{14}C -TNT-amended Burbank soil and control Burbank soil in the same growth chambers. Supplemental nitrogen was withheld (plain soil), given as fertilizer, or given as a *Rhizobia* inoculum. Plants growing in the plain soil were very small in stature after 45 days when compared to the supplemental nitrogen treatments further demonstrating the natural nutrient deficiency of the soil. These plants also exhibited high tissue specific activities in their roots in excess of 14,000 TNT-equivalents/g dry wt. perhaps suggesting a nutrient stress driven tendency to acquire alternate nitrogen sources from the soil. This data must be taken with caution, however, because the average root dry weight for these plants was 0.02 g and the total activity found in these organs averaged 241 μg ^{14}C -TNT-equivalents compared to the 877 and 927 μg ^{14}C -TNT-equivalents for the *Rhizobia* and fertilizer plants, respectively.

There was no TNT toxicity apparent in the larger plants provided with a supplemental nitrogen source (Table 3.14). The added TNT nitrogen may have promoted dry matter accumulation. This observation complimented similar effects observed in the TNT partitioning study for the carrot, spinach, and alfalfa. No significant differences were evident between the supplemental nitrogen treatments in either dry weight, specific activity, tissue contents, or distribution patterns. Therefore, it appears that while some evidence exists for a TNT stimulation of growth in situations where the nitrogen content of the soil may be limited, definitive proof was not obtained. Additional studies employing plants grown to the reproductive and mature stage may be needed to effectively resolve the question.

4.4 RESEARCH NEEDS

The observations made during this study confirm those performed in the previous work with wheat, grass, and bean. They demonstrate that crop plants are capable of accumulating the munitions material and partitioning the material itself, or its metabolites within tissues directly consumed by herbivores or by humans. The information presented here suggests that the amounts of these parent compounds, or their metabolites, may reach significantly high levels (e.g. >180 $\mu\text{g/g}$ RDX in alfalfa shoots) even after the plant has been exposed to the material for over 120 days.

No information exists as to the toxicity of these potentially bioavailable forms to the primary and secondary consumers. Dose response, or species sensitivity, and toxicity information is also unavailable. A study, or series of studies, with these and related munitions compounds to assess their relative toxicity to herbivores and omnivores could provide essential baseline environmental information. Data currently available is vague and not readily transferrable to a field situation. This additional baseline information would therefore be crucial for the accurate performance of any risk analysis, even a critical needs-based evaluation,

- required by federal regulations. This is significant because current knowledge may force any environmental risk evaluation to be excessively conservative and, therefore, severely restrict remediation options on the part of the U.S. Department of Defense with a concomitant increase in the final costs.

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